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Supplemental Data

A *C. elegans* Model of

Nicotine-Dependent Behavior:

Regulation by TRP-Family Channels

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Supplemental Experimental Procedures

Cell Culture and Calcium Imaging

HEK293T cells were maintained in MEM (10% FBS and 5% CO_2). Plasmids encoding TRP-2 cDNA (pcDNA3-TRP2) and YFP (pEYFPc1, Clontech) were transfected into 293 cells with Fugene 6 (Roche). At 36-48 hours after transfection, cells were loaded with 2.5 μM fura 2-AM in ECM (14 mM Hepes [pH 7.4], 4 mM KCl, 145 mM NaCl, 1.2 mM $MgCl_2$, 5 mM $CaC1_2$, and 10 mM glucose) for 30 minutes at room temperature. EGTA (1 mM), Ba^{2+} (5 mM) and Sr^{2+} (5 mM) was used to replace calcium in ECM to make calcium-free, Ba^{2+} and Sr^{2+} solution, respectively. Calcium imaging was performed on a Zeiss Axiovert 200 microscope under a 40x objective. Images excited at 340 and 380 nm with a Xenon source were acquired with a Roper CoolSnap CCD camera and processed by the software Ratiotool (ISeeimaging, Co.) and CoolRatio (home-developed). Only YFP positive cells were selected for analysis.

Calcium imaging of live worms was performed as previously described (Li et al., 2006). G-CaMP and DsRed2 (Clontech) driven by the *nmr-1* promoter were co-expressed as a transgene, allowing for ratiometric imaging. G-CaMP and DsRed2 fluorescence was excited at 484 nm and 565 nm, respectively. G-CaMP, but not DsRed2, responds to calcium change (Li et al., 2006; Nakai et al., 2001). The percentage ratio change (484/565 nm) was thus utilized to quantify the relative change of calcium levels in the neuron. Animals were glued laterally on a 2% agarose pad with Nexaband cyanoacrylate glue (Fisher) in ECM. Nicotine-containing ECM (100 μM) was perfused toward the worm to elicit calcium responses in AVA.

Behavioral Quantification

After being transferred to a new environment (i.e. a fresh plate), the animal typically exhibits an exponentially decay in locomotion speed (V_t) until reaching a relatively steady state (Zhao et al., 2003). This process can be readily quantified by the following equation 1, which is adapted from Zhao et. al (Zhao et al., 2003). This equation can also be used to quantify the behavioral state during which worms speed up their locomotion (see below). With this method, the allowable error of kinetic fitting can be controlled within 0.1%.

$$v_t = m1 + m2 \times \exp(pt) \qquad \text{equation 1}$$

To obtain meaningful kinetic fitting, centroid velocity data from 5-20 worms were grouped and averaged at each time point. The solid line running through the trace in Figure 1A-C, 1D, 2A-B represents the fitting line (see below).

{The following section describes how equation 1 is derived: Similar to other biological systems exhibiting exponential changes, the change of worm locomotion velocity within a given time period (Δt) can be characteristically quantified by the proportion (*p*) of the changed value (ΔS) to the current value (S) [see reference (Edelstein-Keshet, 2005)]

$$p(t) = (\frac{\Delta S}{S_t}) / \Delta t \qquad \text{equation 2}$$

When the animal speeds up its locomotion, the value of ΔS and *p* is positive. Conversely, ΔS and *p* are negative when the animal reduces its speed. If the animal maintains a steady velocity, ΔS and *p* are then equal to zero. We consider the simplest situation in which *p* is a constant during the time period:

$$\frac{dS}{dt} = pS \qquad \text{equation 3}$$

Therefore, at a given time point t from time 0:

$$S_t = S_0 \times \exp(pt) \qquad \text{equation 4}$$

in which *p* and S_0 are constants. S_t is the speed at time t, and S_0 is the speed at time 0. Because animals may reach a steady velocity or a limit (m1), we replace S_t with v_t – m1, in which v_t is the velocity at time t. Therefore equation 4 becomes (m1 and m2 are constants):

$$v_t = m1 + m2 \times \exp(pt) \qquad \text{equation 1}$$

In equation 1, v_t is the velocity at time t; m1 is the limit; and m2 is the changing amplitude (v_0 – m1). τ (half-life) and *d* (doubling time) are commonly used to quantify linear differential mathematical models of biological processes, and their relationship to *p* is described in equation 5.

$$\tau = {\ln(0.5)}/{p} \text{ or } d = {\ln(2)}/{p} \qquad \text{equation 5}$$**}**

In the case of naïve or nicotine-adapted animals, the locomotion velocity vs. time can be well fitted into equation 1 with a single *p* value during our monitored time period (16 mins). Upon acute nicotine treatment or withdrawal, the initial phase of speed decay observed in naïve or nicotine-adapted animals only lasts a few minutes (~4 mins), and is immediately followed by a locomotion-stimulation phase. Mathematically, this phenomenon can be modeled as a transition between two independent processes. Such a dual-phase phenomenon can be quantified by two *p* constants *p*1 and *p*2, with *p2* being characteristic of the locomotion-stimulation phase. This can be well fitted into a double exponential equation using Igor Pro 5 (WaveMetrics) (p_1, p_2, m1, m2, m3, m4 are constants):

$$v_t = Max(m1 + m2 \times \exp(p_1 t), m3 + m4 \times \exp(p_2 t)) \qquad \text{equation 6}$$

To quantify nicotine-induced locomotion-stimulation, we created a locomotion-stimulation index (*I*):

$$I_{stimulation} = \frac{p_2(nicotine) - p(naive)}{\left|p(naive)\right|}$$ equation 7

The nicotine-withdrawal index (I) was created as follows:

$$I_{withdrawal} = \frac{p_2(withdrawal) - p(adaptation)}{\left|p(adaptation)\right|}$$ equation 8

Supplemental References

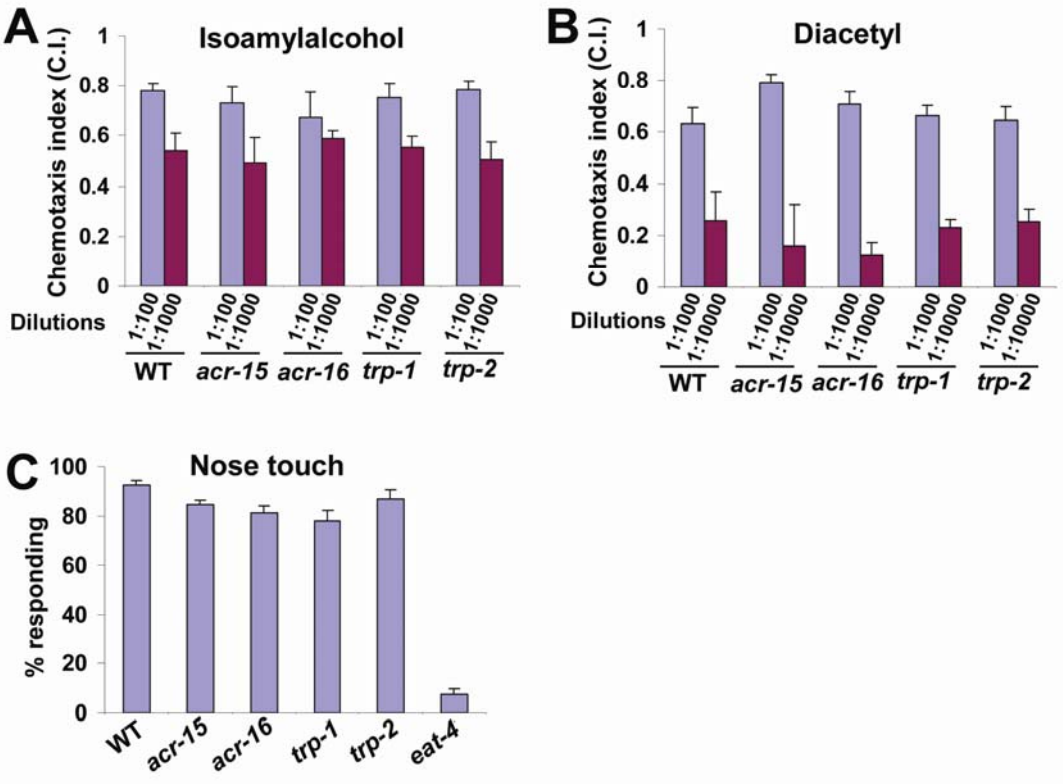
Edelstein-Keshet, L. (2005). The theory of linear difference equations applied to population growth. In Chapter 1 of "Mathematical Models in Biology" (Random House, New York, Society for Industrial and Applied Mathematics (SIAM)).

Li, W., Feng, Z., Sternberg, P. W., and Xu, X. Z. S. (2006). A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. Nature *440*, 684-687.

Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. Nat Biotechnol *19*, 137-141.

Zhao, B., Khare, P., Feldman, L., and Dent, J. A. (2003). Reversal frequency in Caenorhabditis elegans represents an integrated response to the state of the animal and its environment. J Neurosci *23*, 5319-5328.

Figure S1

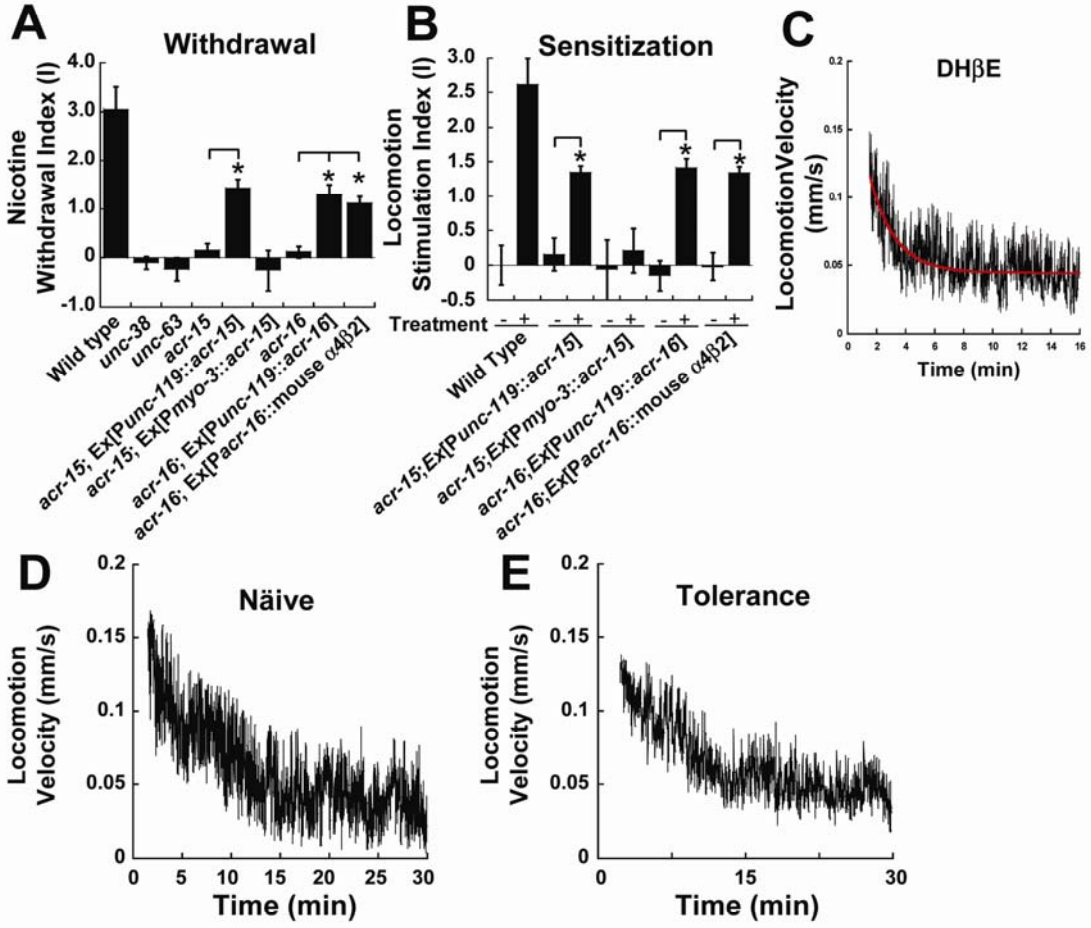


Supplemental Figure S1. No significant defect in chemotaxis or nose touch was observed in *acr-15, acr-16, trp-1* and *trp-2* mutant worms.

(A-B) Chemotaxis assay. Isoamylalcohol and diacetyl were chosen as they are detected by the two pairs of olfactory neurons AWC and AWA, respectively (Bargmann, et al. 1993 *Cell* 13, 515-27). Chemotaxis index was calculated according to the standard protocol (Bargmann, et al. 1993 *Cell* 13, 515-27). Error bars represent SEM. $n \geq 4$.

(C) Nose touch assay. At least 15 animals were tested for each genotype using the standard protocol (Hart, et al. 1995 *Nature* 378, 82-5). Error bars represent SEM.

Figure S2



Supplemental Figure S2. Rescue of nicotine withdrawal and sensitization in *C. elegans* nAChR mutants by neuronal expression of *C. elegans* nAChRs or by expression of mouse nAChR α4β2**.** See Figure 3 for acute response.

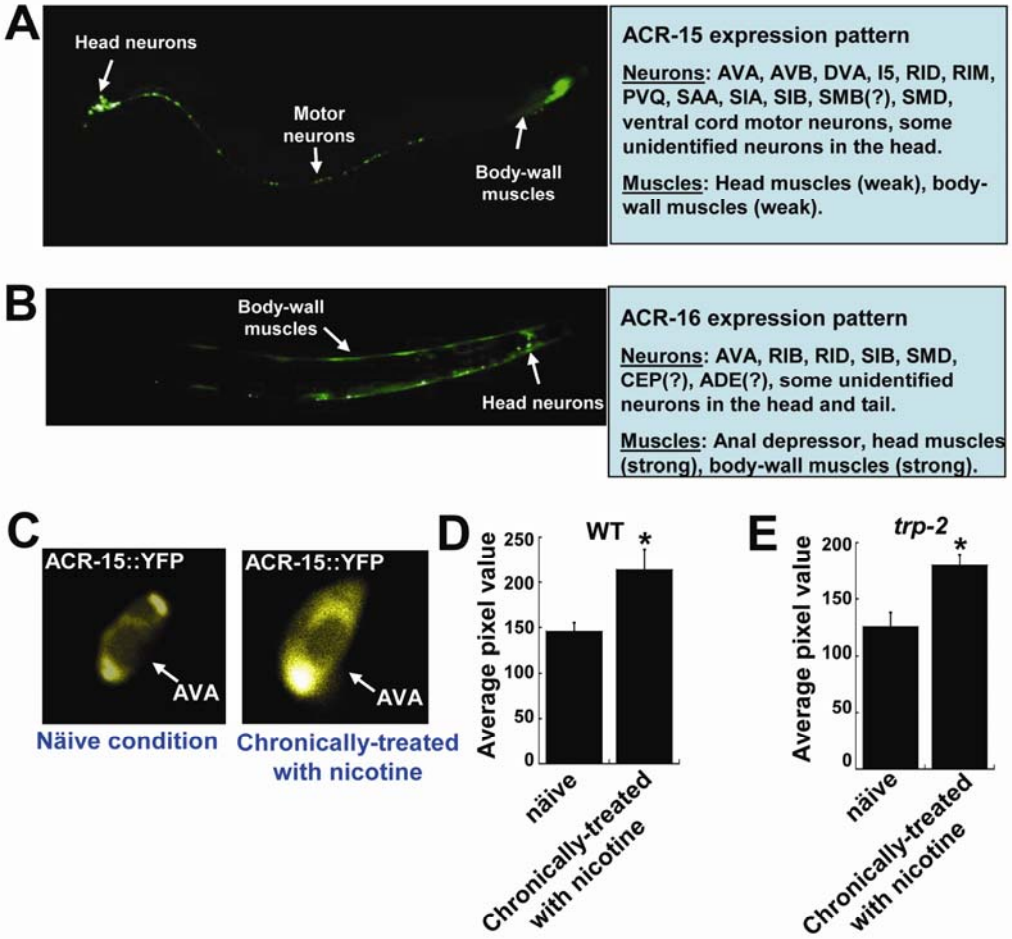
(A) Withdrawal rescue. Genotype descriptions can be found in the legends of Figure 3. Animals treated with 1.5 μM nicotine for 16 hrs were assayed on nicotine-free plates. See experimental procedures for allele names. Error bars represent SD. **p*<0.05 (ANOVA with Kruskal-Wallis H-test).

(B) Sensitization rescue. Animals received 3 doses of intermittent nicotine treatments using the protocol described in the main text. **p*<0.05 (Mann-Whitney U-test).

(C) DHβE does not reduce the basal locomotion rate (see Figure 1A for comparison). Worms were tracked on plates containing 20 μM DHβE.

(D-E) After being transfferred to a fresh plate, worms gradually reduce their locomotion speed until reaching a relatively steady state. Tracking was performed as described for Figure 1A and 1C, except that the worms were tracked for a longer period of time (30 mins vs. 16 mins).

Figure S3



Supplemental Figure S3. *acr-15* and *acr-16* expression patterns.

(A) Expression pattern of *acr-15* determined by a transgene expressing YFP under the *acr-15* promoter (~1.6 kb). Shown in the left panel is a YFP fluorescence image. Cells expressing YFP are listed in the right panel.

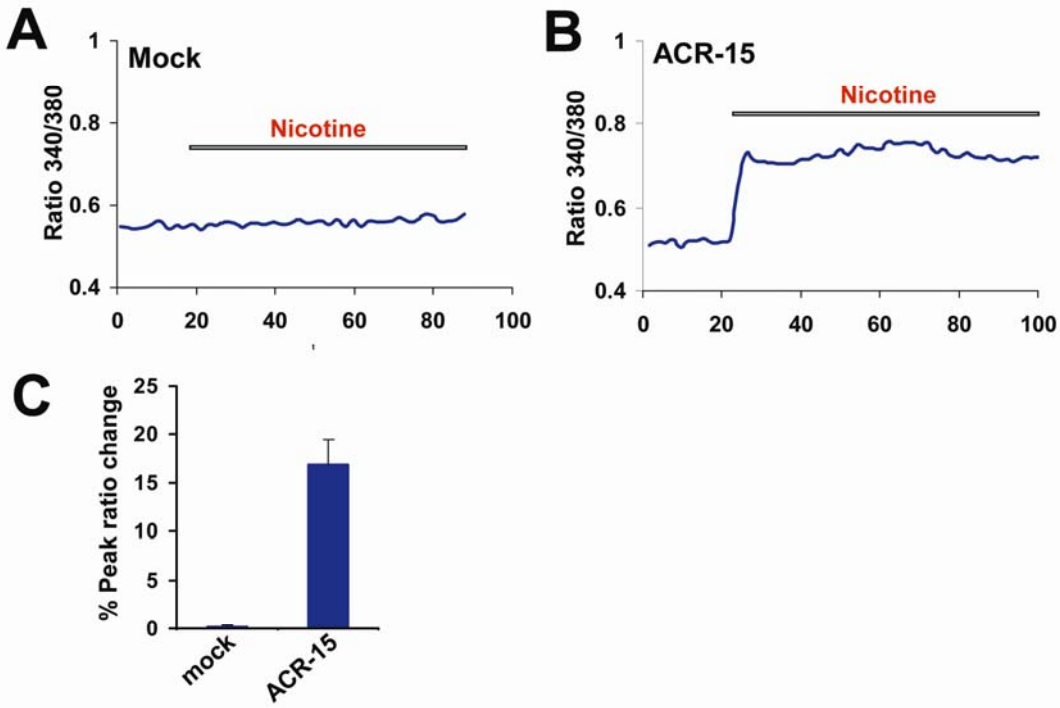
(B) Expression pattern of *acr-16* determined by a transgene expressing YFP under the *acr-16* promoter (~6 kb). Shown in the left panel is a YFP fluorescence image. Cells expressing YFP are listed in the right panel. *acr-16* showed stronger expression in muscle cells compared to *acr-15*.

(C) Chronic nicotine treatment up-regulates ACR-15::YFP expression in the command interneuron AVA. ACR-15::YFP is a functional fusion as it can rescue *acr-15* mutant phenotypes (data not shown). AVA was chosen for analysis because of its relatively easy identification in adult worms, its essential role in nicotine-dependent behavior and its robust calcium responses to nicotine stimulation. Worms were chronically treated with 1.5 μM nicotine for ~16 hours before imaging.

(D) Histogram of ACR-15::YFP expression level in AVA in wild-type worms. *p<0.005 (*t*-test).

(E) Histogram of ACR-15::YFP expression level in AVA in *trp-2* mutant worms. *p<0.005 (*t*-test). The same ACR-15::YFP transgene from (D) was crossed into the *trp-2* mutant background.

Figure S4



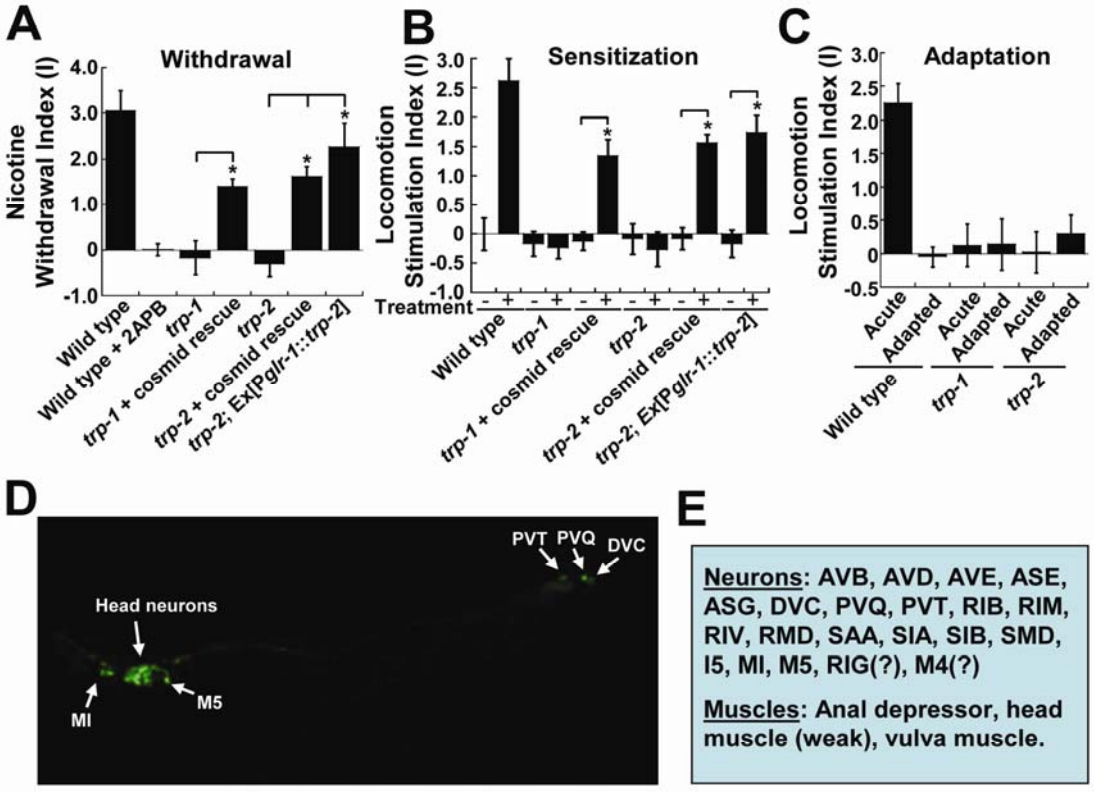
Supplemental Figure S4. ACR-15 can form a nicotine-sensitive nAChR in HEK293T cells. Cells were loaded with the calcium dye Fura-2 AM to permit calcium imaging and kept in ECM solution (see experimental procedures for details). Nicotine (500 μM) was applied to cells to stimulate nAChR activity.

(A) Mock-transfected HEK293T cells do not respond to nicotine. Shown is a representative trace.

(B) Expression of ACR-15 (L9'A) in HEK293T cells make these cells responsive to nicotine. L9'A is a Leu/Ser point mutation introduced into the M2 transmembrane domain (the 9th position) of ACR-15. The same mutation has been found to greatly slow down the desensitization of mouse nAChR channels and also make these channels hypersensitive to nicotine (Labarca, et al. 1995 *Nature* 376, 514-6). The wild-type form of ACR-15 gave rise to little nAChR activity (data not shown), probably because of the low expression level of worm genes in human cell lines.

(C) Histogram. Error bars represent SEM. n$\geqslant$18.

Figure S5



Supplemental Figure S5. Nicotine withdrawal, sensitization and adaptation in *trp-2* mutant animals and TRP-2 expression pattern. See Figure 5 for acute response.

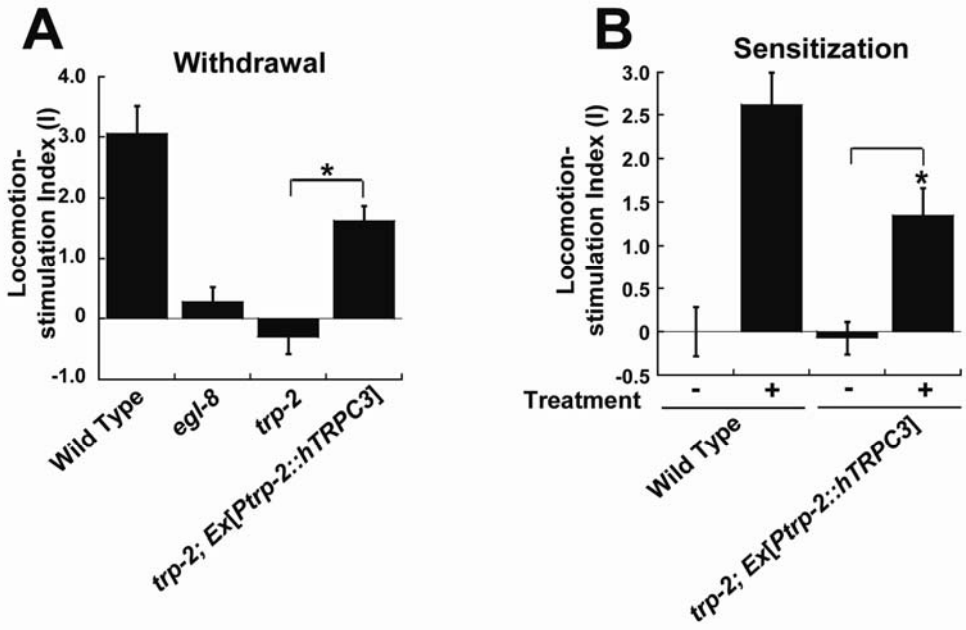
(A) Withdrawal rescue. Genotype descriptions can be found in the legends of Figure 5. Animals treated with 1.5 μM nicotine for 16 hrs were assayed on nicotine-free plates. The effect of 2-APB was tested by moving nicotine-treated wild-type animals to nicotine-free plates containing 50 μM 2-APB. n⩾10. Error bars represent SD. **p*<0.04 (Mann-Whitney U-test)

(B) Sensitization rescue. Animals received 3 doses of intermittent nicotine treatments using the protocol described in the main text. **p*<0.05 (Mann-Whitney U-test)

(C) Adaptation. Worms were pretreated with nicotine (1.5 μM) overnight and then assayed on nicotine plates (1.5 μM).

(D-E) TRP-2 expression pattern. To make transgenic worms expressing *trp-2::yfp*, a ~5kb genomic fragment encompassing the promoter region and the first 128 residues of the *trp-2* gene was amplified by PCR, fused in frame with YFP and injected into the *pha-1(e2123)* background. Shown in (D) is a fluorescent image of a L1 transgenic worm. TRP-2 is widely expressed in the nervous system including interneurons, motor neurons, sensory neurons and pharyngeal neurons, as well as in a few muscle cells. Cells expressing TRP-2 are listed in (E).

Figure S6



Supplemental Figure S6. Rescue of nicotine withdrawal and sensitization in *trp-2* mutant animals by human TRPC3. See Figure 5 and 7 for acute response.

(A) Withdrawal. Genotype descriptions can be found in the legends of Figure 5 and 7. Animals treated with 1.5 μM nicotine for 16 hrs were assayed on nicotine-free plates. n⩾10. See experimental procedures for allele names. Error bars represent SD. **p*<0.05 (Mann-Whitney U-test)

(B) Sensitization. Animals received 3 doses of intermittent nicotine treatments using the protocol described in the main text. **p*<0.05 (Mann-Whitney U-test)